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Introduction

The overall goal of this proposed project is to understand the mechanism of regulation of human BRCA2 gene expression in order to explore the possibility of epigenetic malfunction in this mechanism, which may lead to sporadic breast cancer. The majority (>95%) of human breast cancer happen sporadically and caused by mutations in a variety of genes (1-5). On the other hand, the familial breast cancers are caused by the defects in either of the two DNA repair protein genes, BRCA1 and BRCA2 (1). Possibility of epigenetic malfunction in the expression of these genes in developing sporadic breast cancer has been proposed. BRCA2 mRNAs were only detected in dividing cells but not at all in quiescent cells (1-5). Recently, we have found an Alu-repeat containing transcriptional silencer at the upstream of human BRCA2 gene (6). This silencer is active only in the quiescent cells but not in the dividing breast cells, thus explaining the absence of BRCA2 mRNA in the quiescent cells. The mechanisms of the activation and inactivation of this silencer in the quiescent and dividing cells, respectively, are presently unknown. We have shown that specific nuclear proteins from quiescent breast cell nuclear extract sequence-specifically binds to this silencer (6). Understanding the structure-activity relationships in these bindings in reference to covalent modifications of the DNA elements and the protein factors may reveal the mechanisms of the regulation of the silencer function. Thus, we believe that the human BRCA2 gene is silenced in the quiescent stage of breast cells but is activated in the dividing cells by the inactivation of an Alu-containing silencer located at the upstream of the BRCA2 gene promoter. Possible transient epigenetic malfunction in this silencer inactivation process by environmental factors in the dividing cells may lead to defect in DNA repair and subsequent onset of mutations in any key gene leading to oncogenesis. Since there are indications that the development and progression of breast cancer in African Americans may be different from that of Caucasians (7-16), we planned to explore whether the BRCA2 silencer turn-on and turn-off mechanisms are altered in the breast cells isolated from African American females.

Body

Task 1: To evaluate further the function of BRCA2 gene silencer and the silencer binding protein(s) in human breast epithelial cells of different ethnic origin.

- **Test various human breast cell lines for their BRCA2 gene silencer (221 bp) activity:** The map of the human BRCA2 gene transcriptional silencer is shown in Fig. 1. We made constructs (Fig. 2) to test the activity of this silencer in several lines of human breast cells of African American and Caucasian origins (Table 1). The cells were serum-starved to take them to the quiescent stage. In a parallel study, we found that the silencer works only at the quiescent stage of the cells. It is inactive in the dividing cells. With the exception of MDA-MB-157, the cells of African American origin showed less activity of the silencer as compared to the cells of Caucasian origin. Table 1 shows the silencer activities with the SV40 promoter/enhancer system. Similar activities were found with the BRCA2 promoter/enhancer system (Fig. 2). Some of cells of African American origin multiply poorly in the growth medium prescribed by ATCC and we could not store them in liquid nitrogen. Thus, among the cells of African American origin listed in Table 1, we worked only with MDA-MB-157, Hs 275.T, and MDA-MB-468 cells.

Table 1. BRCA2 gene silencer (221 bp) activity in transiently transfected human breast cells of different ethnic origins (data from the SV40 promoter/enhancer system are shown)

Cell line Name	ATCC #	Breast Cell Nature	Silencer Activity ^a
African-American origin (Black female)			
Hs 927.T	CRL-7679	Fibrocystic disease*	32 ± 3
MDA-MB-157	HTB-24	Breast carcinoma	69 ± 1
Hs 275.T	CRL-7224	Breast carcinoma	27 ± 3
MDA-MB-468	HTB-132	Adenocarcinoma	13 ± 2
Hs 496.T	CRL-7303	Breast carcinoma	44 ± 6
Hs 467.T	CRL-7809	Breast carcinoma	41 ± 5
Caucasian origin (White female)			
MCF-10F	CRL-10318	Fibrocystic disease*	62 ± 7
MCF-7	HTB-22	Adenocarcinoma	81 ± 4
MDA-MB-231	HTB-26	Adenocarcinoma	72 ± 3
MDA-MB-361	HTB-27	Adenocarcinoma	67 ± 8
MDA-MB-436	HTB-130	Adenocarcinoma	73 ± 1
MDA-MB-453	HTB-131	Breast carcinoma	86 ± 4

*'Normal' breast cells; ^aSilencer activity in the cells transfected with the silencer containing reporter plasmid (Fig. 2) is expressed as % inhibition of the normalized luciferase activity in cells transfected with the control plasmid without the silencer. Values are Mean ± SE (n=6).

Similar results were obtained with BRCA2 promoter constructs.

Task 2: To identify the sequence elements in the BRCA2 gene silencer responsible for the activity of the silencer by mutational analysis.

Purify the silencer binding proteins (BSBPs) from breast cell nuclear extracts: We previously reported binding of proteins of the quiescent breast cell nuclear extract to the silencer sequence in *in vitro* EMSA analysis (6). Three to five DNA-protein complexes were formed (6). These results suggest that the binding of proteins to the E2 box sequence is a primary event for the formation of all the DNA-protein complexes. Use of Alu-repeat-less sequences as probes for EMSA showed only few [1-2] lower molecular size complexes (not shown). On the other hand, use of Alu sequence alone did not bind any protein from the quiescent cell nuclear extract (not shown). Alu sequences alone did not compete the binding of the proteins to the 221 bp silencer sequence probe (not shown). Substitution of the mutated do-decamer in the experiment in lane 4 with mutated BRS5 as competitor, did not change the result, suggesting that the pseudo-B-box sequence may not have any role in the binding of quiescent cell nuclear proteins to the E2-box of the silencer, at least *in vitro*.

We attempted to purify the primary binder proteins to the inter-Alu sequence of the silencer using DNA affinity chromatography (20). Complementary strands of a 63-bp BRCA2 silencer genomic fragment from the inter-Alu region, 5'-AGGCAGATCACCTGAGGTC GGGAGGTTGAGACCAGACTGACCAACAACGGAGAAACCCCGTCT-3', which included

a stretch of six nucleotides of the E2 box sequences, were synthesized to contain five tandem copies with an *Eco*RI site at the 5' end and *Xho*I and *Xba*I sites at the 3' end. We custom synthesized the 63-mer and gel purified. We then mixed this oligo with a 33-mer chimeric oligo 5'-CCTCAGGTGATCTGCCTAGACGGGGTTTCTCCG-3', to anneal following standard protocol (97) the 3'-end of one molecule of the 63-mer with the 3'-end of a 33-mer molecule and the 5'-end of another 63-mer molecule with the 5'-end of the same 33-mer molecule. We added T4 DNA ligase to tandemly ligate the 63-mer oligos to form multimers. We then PCR amplified the multimers of the 63 bp tandem repeats using the *Eco*RI-site anchored forward primer 5'-CCCCCGAATTCAGGCAGATCACCTGGGTCGG-3' and *Xho*I/*Xba*I site anchored reverse primer 5'-CCCCCTCGAGTCTAGACAGACGGGG TTTCTCCG-3'. The five tandem repeat of ~350 bp was gel purified and cloned into pCR 4.0 Topo vector (Invitrogen). The nucleotide sequence of the pentamer was verified by automated sequencing. The insert was cut out with *Eco*RI/*Xho*I and the gel-purified DNA was end labeled with biotin by fill-in reaction using biotin-11-dUTP (Invitrogen), other dNTPs and Klenow polymerase following standard protocols (20). Biotin label from one end of the DNA was removed by the digestion of the purified biotin-labeled DNA with *Xba*I. Nuclear proteins of the quiescent (16 h) or dividing (6 h) BT549 cells were radiolabelled with ³⁵S-methionine following standard protocols (20). The proteins from the nuclear extract (20 mg/ml) were allowed to bind (1 h, 4°C) to the high-affinity recognition sites present in the biotinylated DNA fragment. Streptavidin (Caltech Ltd) was then added to the biotin labeled DNA to bind to the biotinylated end of the DNA fragment. Next, the protein/biotinylated fragment/streptavidin ternary complex was removed by adsorption onto a biotin-containing resin, biotin cellulose (Pierce). Since streptavidin is multivalent, it was able to serve as a bridge between the biotinylated DNA fragment and the biotin-containing cellulose. The resin was washed 3x with the biotin-cellulose binding buffer (12% glycerol, 12 mM Hepes-NaOH, pH7.9, 4 mM Tris-Cl, pH 7.9, 60 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol) to remove loosely bound proteins. Finally, the proteins tightly bound to the DNA were eluted from the resin with the elution buffer (12% glycerol, 20 mM Tris-Cl, pH 6.8, 1 M KCl, 5 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol) following the protocol as described (20). The proteins were concentrated using Centricon columns and analyzed by 10% SDS-PAGE followed by autoradiography. Fig. 6 shows the autoradiogram of typical preparations from quiescent and dividing cells. A ~29 kDa protein is predominantly purified from the quiescent cell nuclear extracts. There is also a ~125 kDa protein in this purified fraction. The sizes of the proteins purified correspond to those of Snail and ZEB family of repressor proteins. The predominant protein that is purified from dividing cell nuclear extract was of ~23 kDa. There was also a minor band at ~29 kDa. The ~23 kDa protein band corresponds to the predicted size of ACR1. Other than the sizes, we do not have any evidence for the identities of the purified proteins (Fig. 3). We are currently optimizing the wash and elution conditions. Use of a mutated E2-box containing 63 bp ligand (m1BRS5, 5'-AGGCAGATAACCTAAGGTCGGGAGGTTGAGACCAGACTGACCAACAACGGAGAAACCCCGTCT-3') made similar way, failed to purify any protein from quiescent BT549 cell nuclear extracts but could pull down the ~23 kDa protein from the dividing cell nuclear extracts.. Similarly, a probe with altered pseudo B-box sequence (m2BRS5, 5'-AGGCAGAT CACCTGAGGTCGGGAGACCTCAACAAGACTGACCAACAACGGAGAAACCCCGTCT-3') still purified the ~29 kDa protein from quiescent cell extracts but did not purify any ~23 kDa protein from dividing cell nuclear extracts. We will separate these proteins by 2-D gel electrophoresis and subject the separated proteins to limited proteolysis, separation of the fragments by HPLC and determine the amino acid sequences of the

peptide fragments to verify the identity of these proteins. Although we do not have any evidence yet, we believe that the binding of mediator proteins to the E2-box-bound repressor may need the Alu sequences around the E2-box.

Site-directed mutagenesis of the potential cis-elements in the BRCA2 silencer: The QuikChange site-directed mutagenesis kit (Stratagene) was used. We designed the mutagenic primers according to the instruction given by Stratagene in their manual to alter key nucleotide sequences known to be essential for the functions of Alu and E-box elements. The lists of the mutagenic primers are given in Table 2. The 221 bp silencer was cloned into pCRII vector (Invitrogen) for mutagenesis. After mutagenesis the insert in the mutated plasmid DNA was sequenced to verify mutagenesis and then the mutated silencer was subcloned into the reporter vector (pGL3; see Fig. 2). We are successful in obtaining four single mutants, six double mutants and one mutant having all the four cis-elements mutated (see Table 3 for their names).

Evaluation of protein binding to the mutated silencer: We determined the binding of breast cell (MDA-MB-231) nuclear proteins to the silencer and determined the competition of those bindings with wild-type and mutated cis-element sequences (ds-DNA composed of annealed oligos as detailed in Table 1). While Alu1 or Alu2 sequences did not show any detectable competition of protein binding to the silencer (data not shown), E1 and E2 sequences competed for the bindings (Fig. 3). Mutated E-box sequences failed to compete (Fig. 3) for the binding, suggesting that these elements are important for the protein binding at least in vitro. We have repeated this experiment with nuclear extracts from MDA-MB-468, BT-549 and MCF-7 cells which gave us similar data (not shown).

Evaluation of the silencer activities of the mutants: We have tested the activities of the control and mutated silencer sequences against SV40 promoter/enhancer or BRCA2 promoter/enhancer systems (Fig. 2). Our results are summarized in Table 2. Mutations of either of the Alu elements abrogated the silencer activity somewhat. On the other hand mutations of either of the E-box sequences alone did not have any effect on the silencer activity. Mutants containing Alu1+E2 or Alu2+E1 mutations were found to be largely inactive as silencer. Mutations in all the four cis-elements completely abrogated the silencer activity (Table 2).

Task 3: To determine the influence of cell cycle stages and estrogen on the differential activity of this silencer and the levels of silencer binding proteins in human breast epithelial cells.

Development of permanent transfectants of breast cells expressing luciferase from promoters (SV40 or BRCA2) with or without the 221 bp silencer: Human breast cell lines we used in this study include MCF-10A, MDA-MB-231, MDA-MB-435S, BT-549, and MCF-7. HeLa (human cervical carcinoma), U937 (human histocytic lymphoma), and HepG2 (human hepatocellular carcinoma) cell lines are used as non-breast control cells. These cells were originally procured from American Type Culture Collection (ATCC) and maintained in the recommended (by ATCC) growth medium under the specified conditions. We also use human mammary epithelial cells (HMEC) cells obtained from Clonetics (BioWhittaker, Walkersville, MD). These normal human breast cells are maintained in the MEGM medium (Clonetics). Preliminary identification, cloning and characterization of the human BRCA2 silencer by

deletion mutagenesis have been published (6). Please find the enclosed reprint in the Appendix materials. Relative location of the silencer element with respect to the basal BRCA2 gene promoter is shown in Fig. 1. The nucleotide sequence of the 221 bp silencer is shown in Fig. 1B. The alignment of the two 55 nt direct Alu repeat is shown in Fig. 1C. There is about 82% sequence identity. The inter-Alu repeat sequence contains the E2-box and the putative ACR1 binding site (Figs. 1). The alignment of the inter-Alu repeat sequence (63 nt) with the ACR1 binding site sequence (12) is shown in Fig. 1B. There are >83% identity in these sequences.

We previously have shown the activities of the silencer in human breast cells by transient transfection assays (6). The role of chromatin in down-regulating transcription has been well characterized (17). In view of the fact that small, transiently transfected plasmids may not acquire higher order levels of chromatin structure (17) and thereby allow activity in a nonpermissive cellular milieu, we reason that stable transfectants might potentially represent a more appropriate experimental system. We have developed two sets of plasmid constructs: (i) One set based on pGL3-control Vector (Promega) with or without the silencer sequence cloned in natural orientation with respect to the SV40 promoter (Fig. 2A); and, (ii) the other is based on pGL3-basic vector (Promega) with the human BRCA2 basal promoter with or without the silencer sequence cloned in natural orientation with respect to the promoter (Fig. 2B). We transformed BT549 cells with these plasmids individually along with pcDNA 3.1 plasmid (Invitrogen) as described previously (6), and selected for G418-resistant (200 µg/ml) cells to obtain stably transfected cells. These cells were serum starved for 40 h (at 0.5% serum) to obtain quiescent cells similar to as described for MCF-7 cells (18).

Synchronization of human breast cell populations: Continuous incubation of the serum-starved cells with 10% serum containing medium gave us dividing cells. Flow cytometric analysis of propidium iodide-stained cells revealed >90% of the serum-starved cells are in G0/G1 phase. We assayed the extracts of these cells in passive lysis buffer (Promega) for luciferase activity using LARII reagents in a luminometer (Turner Design), as described before (6). Protein contents of the extracts were assayed using RC/DC reagents (BioRad). Luciferase activities were expressed as LU/µg cellular protein. Results are tabulated in Fig. 2. SV40 promoter/enhancer was relatively more active in the BT549 cells as compared to the BRCA2 promoter (Fig. 2). In both cases, the silencer was only active in the quiescent stage of the cells and inactive in the dividing cells (Fig. 2). Incubation of cells with the histone deacetylase (HDAC) inhibitor trichostatin A (Sigma) at 300 nM inhibited 80% of the silencer activity at the quiescent stage suggesting involvement of HDAC in this process.

Evaluation of the silencer activity in human breast cells at different cell cycle stages: To understand the structure-activity relationship with the BRCA2 silencer, we created additional four different constructs with the BRCA2 basal promoter system, as described in Fig. 2. Since the silencer seems to have four different potential regulatory sequences, i.e., Alu1, Alu2, E2-box and the putative ACR1 binding sequence, we one-by-one replaced or mutated those sequences of the silencer to test the role of each of those elements in the silencer function in a stable transfection system. We replaced the 55 bp Alu1 or Alu2 or the 49 bp putative ACR1 binding sequence with same length of a sequence from pBluescript KS (+) phagemid by overlap extension PCR (19). We also have mutated the E-box sequence from 5'-CACCTG-3' to 5'-AACCTA-3' using Stratagene Quick-Change mutagenesis reagents and mutagenic primers following the manufacturer's recommended protocols. We found that replacement of either of

the Alu sequences abrogated the silencer activity (Fig. 4). Replacement of the putative ACR1 binding site did not have any significant effect on the silencer activity in the quiescent cells but the desilencing of the promoter in the dividing cells are blocked (Fig. 4). E2-box sequence was found to be essential for the activity of the silencer in the quiescent cells (Fig. 4).

Lambda ZAP Express clones containing cDNAs for the silencer E-2 box binding proteins were isolated from a cDNA expression library made from the human breast carcinoma cell MDA-MB-435S (Stratagene). The binding mix for South-western screening of the library replica filters contained 30 ng/ml of ^{32}P -end-labeled (10^8 cpm/ μg) pentameric oligonucleotide BRSE5, 30 $\mu\text{g}/\text{ml}$ of the duplex poly(dI-dC), 10% Ficoll, 100 $\mu\text{g}/\text{ml}$ BSA, 1 mM EDTA, 100 μM ATP, 20 mM HEPES buffer, pH 7.9 and 50 mM NaCl. Incubation was for 1 h at room temperature, filters were then washed for 15 min at room temperature in three changes of the buffer containing 25 mM Tris/HCl, pH 8, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (TNE) and autoradiographed. Before incubation with the labeled DNA probe, filters were preincubated for 30 min at room temperature in TNE plus 5% nonfat dry milk and then washed in TNE. Secondary and tertiary screenings of 33 primary clones, rescue of the pBKCMV clones with EXASSIST helper phage followed by partial nucleotide sequencing and analysis of the cDNA inserts suggested 17 'true' positive clones. Three of the positive clones were for h*Snail*, nine were identical with human *Slug*, and five shared homology with ACR1.

Full-length cDNAs for human SNAIL and ZEB family of proteins were amplified from BT549 cell RNA by reverse transcription-PCR, and a COOH-terminal flag epitope tag was added by PCR (19, 20). Constructs were subcloned into the retroviral expression vector pRevTRE (BD Biosciences Clontech). The identities of all plasmid inserts and vector boundary regions were confirmed by sequence analysis. RtTA protein-expressing retroviruses were generated by transfecting pRevTet-ON plasmid into RetroPack PT67 packaging cells (BD Biosciences Clontech) by electroporation (20). Snail or ZEB protein expressing retroviruses were similarly produced using pRevTRE vector constructs. After transfection (24 h) into the packaging line, the medium was changed and an additional 24 h later virus-containing supernatant was harvested. The supernatant was filtered and diluted 1:1 with fresh medium. It was then supplemented with 4 $\mu\text{g}/\text{ml}$ Polybrene (Sigma Chemical Co.) and used to infect BT-549 cells. After infection of BT-549 (48 h), selection was initiated in 0.5 mg/ml Geneticin (Invitrogen Corp.). The G418-resistant BT549 cells were expressing rtTA protein. These cells were then transfected with viruses containing pRevTRE-constructs of the repressor proteins and hygromycin-resistant cells were developed. The established G418/Hygromycin resistant cells were incubated for 48 h in growth medium with or without doxycycline (1 $\mu\text{g}/\text{ml}$) and the levels of the RNAs of the repressor protein and that of BRCA2 were determined by RT-PCR. We used β -actin as a normalization control for the RT-PCR. We have so far partially characterized the Slug gene expression (Fig. 5). Results show that there is 7-9 fold induction of the Slug transcript level in the presence of doxycycline and there was simultaneous reduction of BRCA2 transcripts. Transient transfection of the Slug-overexpressing *dividing* cells with the luciferase reporter constructs described in Fig. 2, showed repressed luciferase activities in the presence of doxycycline but not in its absence. Constructs with mutation at the E2 box did not show such repression (data not shown). These data may indicate inhibition of the silencer by Slug is the cause of decreased expression of BRCA2 in these cells. Further characterizations of these clones are underway. Interestingly, the silencer failed to work in the stationary phases of two human breast cell lines of African-African origins (Fig. 6) and the SLUG gene expressions in these cells are also down (Figs. 7 and 8). We are currently exploring the implications of these observations.

Evaluation of the silencer activities in the presence or absence of added estrogen in the culture medium: BRCA2 gene expression is known to be regulated by estrogen (21-24). We evaluated whether the silencer activity and the levels of silencer binding proteins are regulated by estrogen. We used 17- β -estradiol (Sigma) for this purpose. In some experiments 17- β -estradiol (10 nM) accompanied 10 nM progesterone or 1 μ M trans-4'-hydroxytamoxifen. The steroids were dissolved in absolute ethanol and diluted directly into the medium. Serum used in this study was depleted from endogenous estrogens by dextran coated charcoal (21). Our data (not shown) suggested that there is no apparent effect of 17- β -estradiol on the silencer activity or the binding of proteins to the silencer.

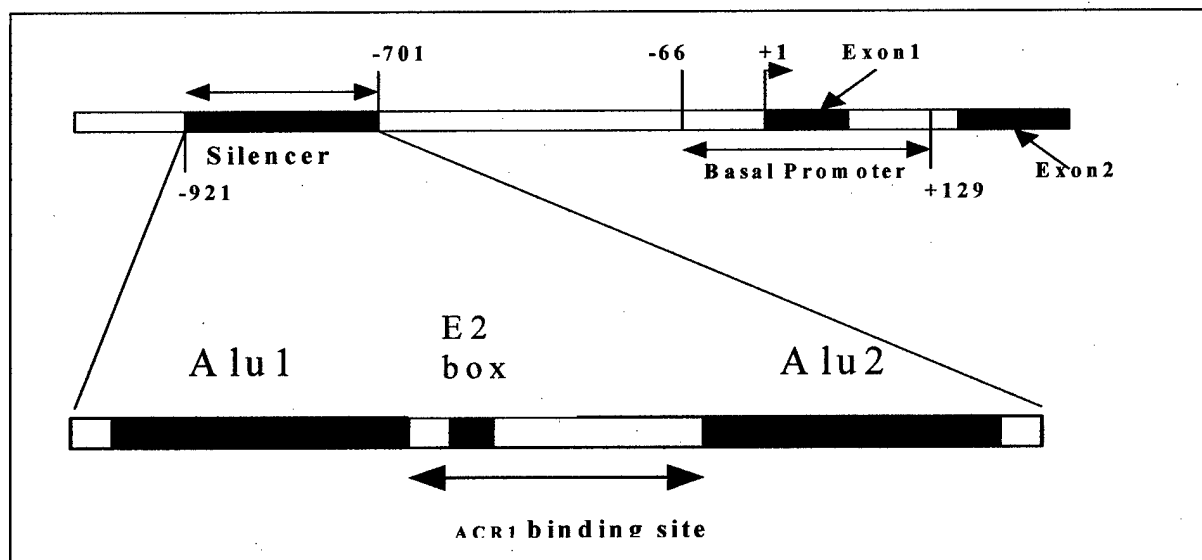


Fig. 1A. Structure and location of human BRCA2 silencer with respect the basal promoter of the gene.

Alu1 **E2-box**

GCAAAAGATGGGCTGGGTGTGGTGGCTCATGCCTGTAATCCCAGCGCTTTGGGAGGCCGAGGCAGGCAGAT**CACCTG**AGGTCGGGAGGTTGAG 93
 CGTTTTCTACCCGACCCACACCACCGAGTACGGACATTAGGGTCGCGAAACCCCTCCGGCTCCGTCCGTCTAGTGGACTCCAGCCCTCCAATC

Alu2

ACCAGACTGACCAACAACGGAGAAACCCCGTCTCTACTTAAAAATGCAAAGTTAGCCGTGCGTGGTGGCCCATGCCTGTATTCCCAGCTACTC 186
 TGGTCTGACTGTTGTGTCCTCTTTGGGGCAGAGATGAATTTTACGTTTCAATCGGCACGCACCACCGGGTACGGACATAAGGGTCGATGAG

GGGAGGCTGAGGCAGGAGAACCACCTTGATCCCTGG 221
 CCCTCCGACTCCGTCTCTTGGTGAAGTACGGGACC

Fig. 1B. Nucleotide sequence of the 221 bp silencer. The 55 bp direct Alu repeats are shadowed. E2-box sequence (CACCTG) is bold-faced and the putative ACR1 binding site (12) is underscored.

A.

Alu1: 5'-GCTGGGTGTGGTGGCTCATGCCTGTAATCCCAGCGCTTTGG**GAGGC**CGAGGCAGG-3'
 Alu2: 5'-GCCGTGCGTGGTGGCCCATGCCTGTATTCCCAGCTACTCG**GAGGC**TGAGGCAGG-3'

B.

BRCA2 Silencer 5'-AGGCAG----AT**CACCTG**AGGTCGGGAGGTT-GAGACCAGACTGACCAACAACGGAGAAACCCCGTCT-3'
 ACR1 bind. Site 5'-AGGCGGGCGGAT**CACCTG**AGGTCAGGAG-TTCGAGACCAGCCTGGCCAACAT-GGTGAAACCCCGTCT-3'
E2-box B Box seq.?

Fig. 1C. Alignments of nucleotide sequences of the Alu1/Alu2 (A) and the inter-Alu sequence/ACR1-binding site (B). The site for the binding of a probable silencer protein (69) is bold-faced and underscored in (A). The E2-box and a pseudo-B-box sequence are underscored in (B).

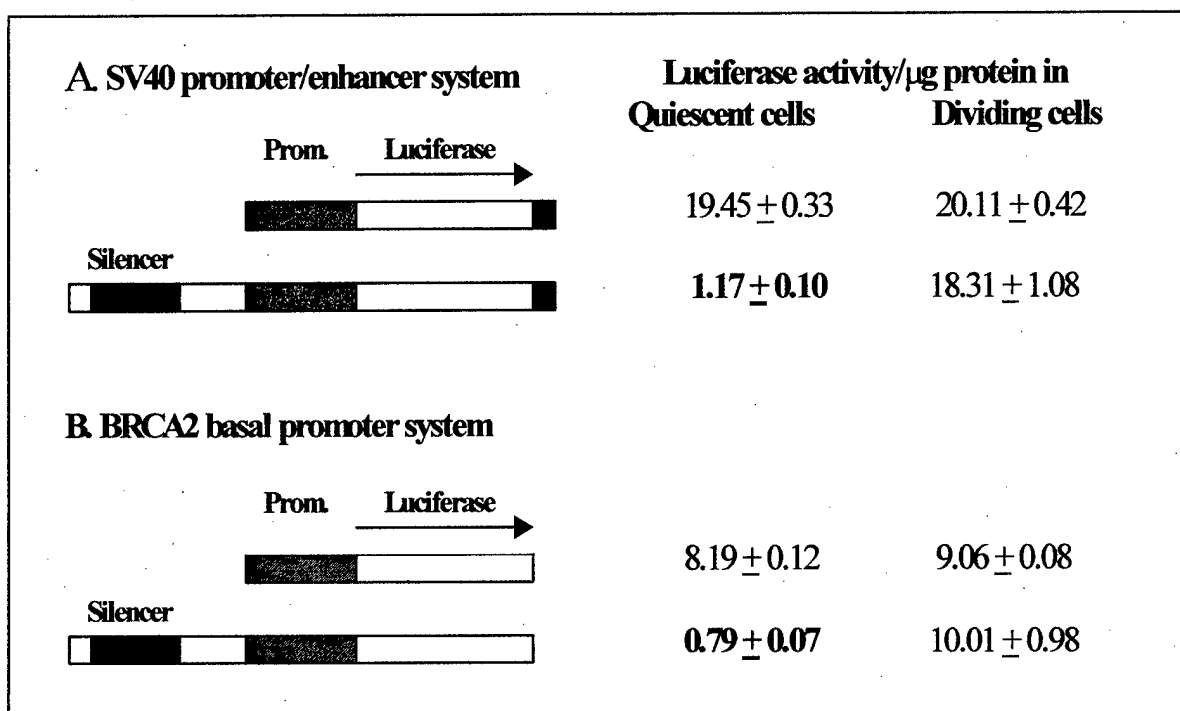


Fig. 2. Activities of the human BRCA2 silencer in stably transfected quiescent or dividing BT549 cells. (A) Repression of SV40 promoter. (B) Repression of BRCA2 basal promoter. The results are mean of six independent assays \pm SE. The luciferase activity was measured as arbitrary light units(LU)/ μ g protein in the cell extract. The differences between the silencer activities (bold-faced) with quiescent and dividing cell extracts were statistically significant ($P < 0.001$). The position or the orientation of the silencer with respect to the promoter has no significant effect on the silencer activity. The vector sequences between the silencer and the promoter had not role in the regulation process.

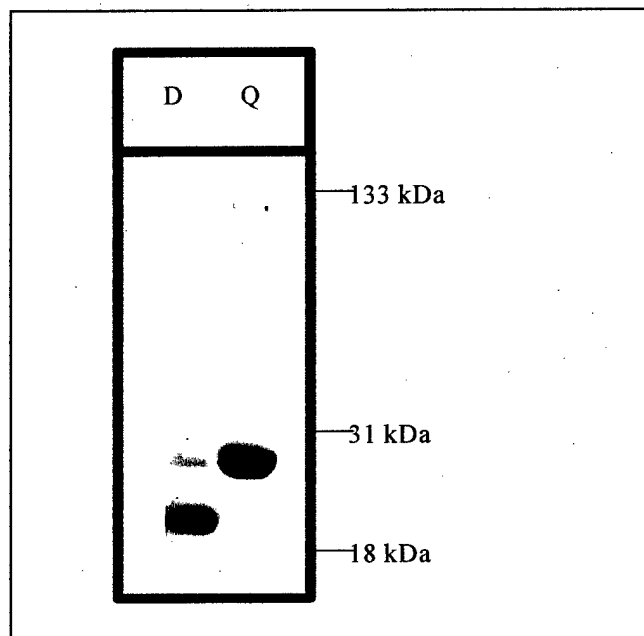


Fig. 3. Affinity purifications of the proteins that binds to the silencer in BT549 cells. SDS-PAGE autoradiogram of the purified proteins from dividing (D) or quiescent (Q) BT549 cells. The autoradiogram was developed after 14 days exposure to X-Ray film with intensifying screen at -80°C .

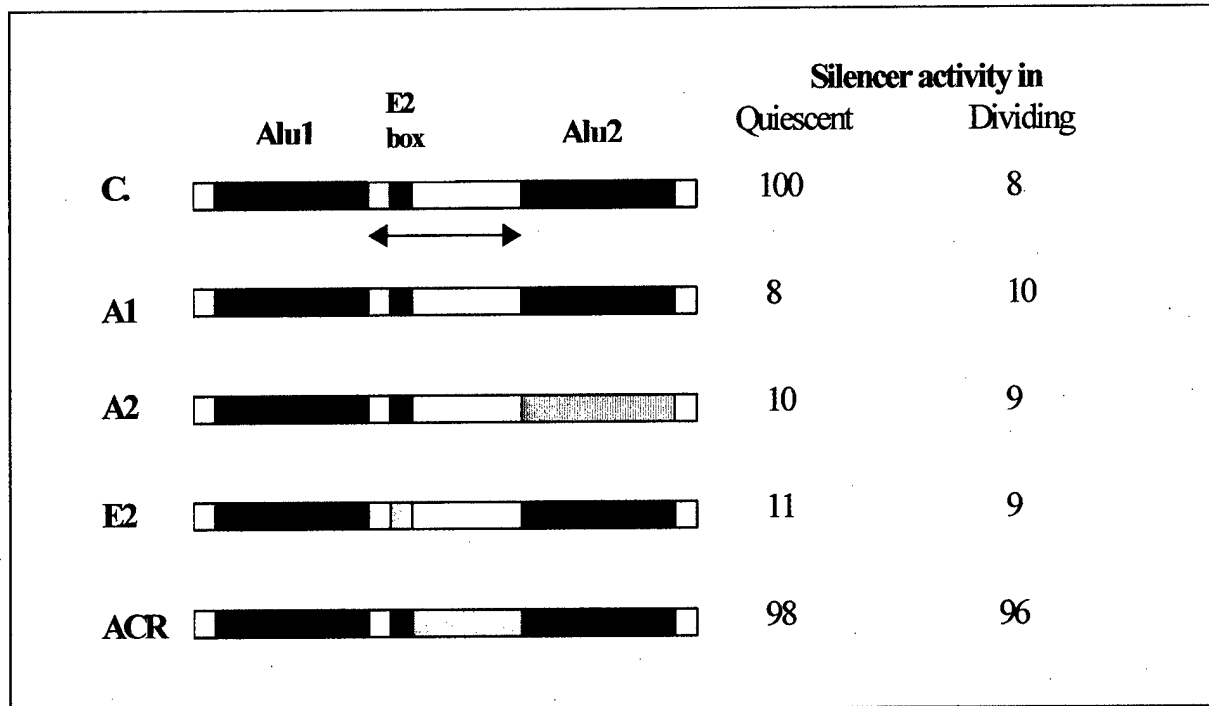


Fig. 4. Structure-activity relationships of the BRCA2 silencer in stably transfected quiescent or dividing human breast cells. Plasmid pGL3-basic (Promega) with BRCA2 basal promoter was used as a control. The left panel shows the structures of the silencer sequences used. Construct 'C' is the un-altered construct. The activity of this silencer in stably transfected quiescent BT549 cells was used as 100 (see Fig. 4). Construct 'A1' has the Alu1 sequence replaced. Construct 'A2' has Alu2 sequence replaced. Construct 'E2' has the mutation in the E2-box and construct 'ACR' has the putative ACR1 binding site replaced. We used two different DNA sequences from pBluescript KS(+) phagemid in each case. Data from one set are shown. The data from the other set were identical.

Table 2. Primers used for Site Directed Mutagenesis (SDM): Mutant nucleotides are in bold and underlined.

E-box 1 (E1) primers	
EB1P1M	5'-GGCAGGAGAACCACT <u>GA</u> ATCCCTGG-3'
EB1P2M	5'-CCAGGGAT <u>TC</u> AGTGGTTCTCCTGCC-3'
E-box 2 (E2) primers	
EB2P1M	5'-CAGGCAGATCAC <u>CGA</u> AGGTCGGGAGG-3'
EB2P2M	5'-CCTCCCGAC <u>CTT</u> CGGTGATCTGCCTG-3'
Alu 1 primers	
Alu1P1M	5'-CCAGCTACTCGG <u>CGAG</u> CTGAGGCAGG-3'
Alu1P2M	5'-CCTGCCTCAG <u>CTCG</u> CCGAGTAGCTGG-3'
Alu 2 primers	
Alu2P1M	5'-CAGCGCTTTGG <u>CGAG</u> CCGAGGCAGG-3'
Alu2P2M	5'-CCTGCCTCGG <u>CTCG</u> CCAAAGCGCTG-3'

Table 3. Effects of mutations in the cis-elements present in the BRCA2 silencer on the silencer activity inside MDA-MB-231 cells. (Data are Mean for four independent experiments \pm SE)

Mutated locus	Silencer activity (% with no mutation)
None	100
Alu1	46.3 \pm 1.7
Alu2	44.2 \pm 2.1
E1	90.1 \pm 2.3
E2	96.2 \pm 1.2
Alu1+Alu2	42.7 \pm 2.5
E1+E2	88.7 \pm 1.3
Alu1+E1	58.1 \pm 1.1
Alu2+E2	61.3 \pm 2.8
Alu1+E2	23.2 \pm 0.9
Alu2+E1	30.2 \pm 1.2
Alu1+Alu2+E1+E2	0.00

Data are shown with plasmid construct B. Similar results were obtained with plasmid construct A.

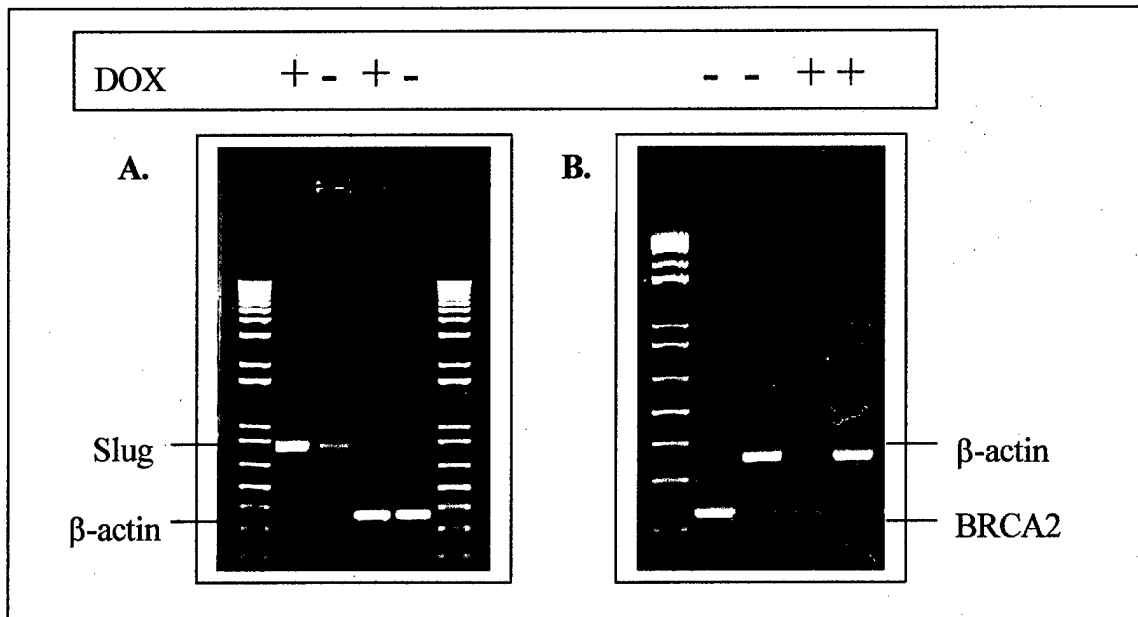


Fig. 5. Over expression of human Slug mRNA in BT549 cells (A) and the effect of this over-expression on the level of BRCA2 mRNA (B). Doxycycline (1 μ g/ml for 48 h) was used for the induction of the gene expression. We have used Amersham one step RT-PCR kit for this experiment. We have used the following primer sets for RT-PCR: Slug: Forward, 5'-GACGGATCCATGCCGCGCTCCTTCCTG-3'/ Reverse, 5'-CGTCGACTCACTTATCGT CGTCATCCTTGTAATCGTGTGCTACACAGC-3'; BRCA2, Forward, 5'-GTACAGGAA ACAAGCTTCTGA-3'/ Reverse, 5'-GACTAACAGGTGGAGGTAAAG-3'; and β -actin, Forward, 5'-GCTCGTCGTCGACAACGGCTC-3'/ Reverse, 5'-CAAACATGATCTGGGTCA TCTTCTC-3'. The sizes of the bands that were amplified by these primer sets are: BRCA2, 225 bp; Slug, 850 bp; and β -actin, 350 bp.

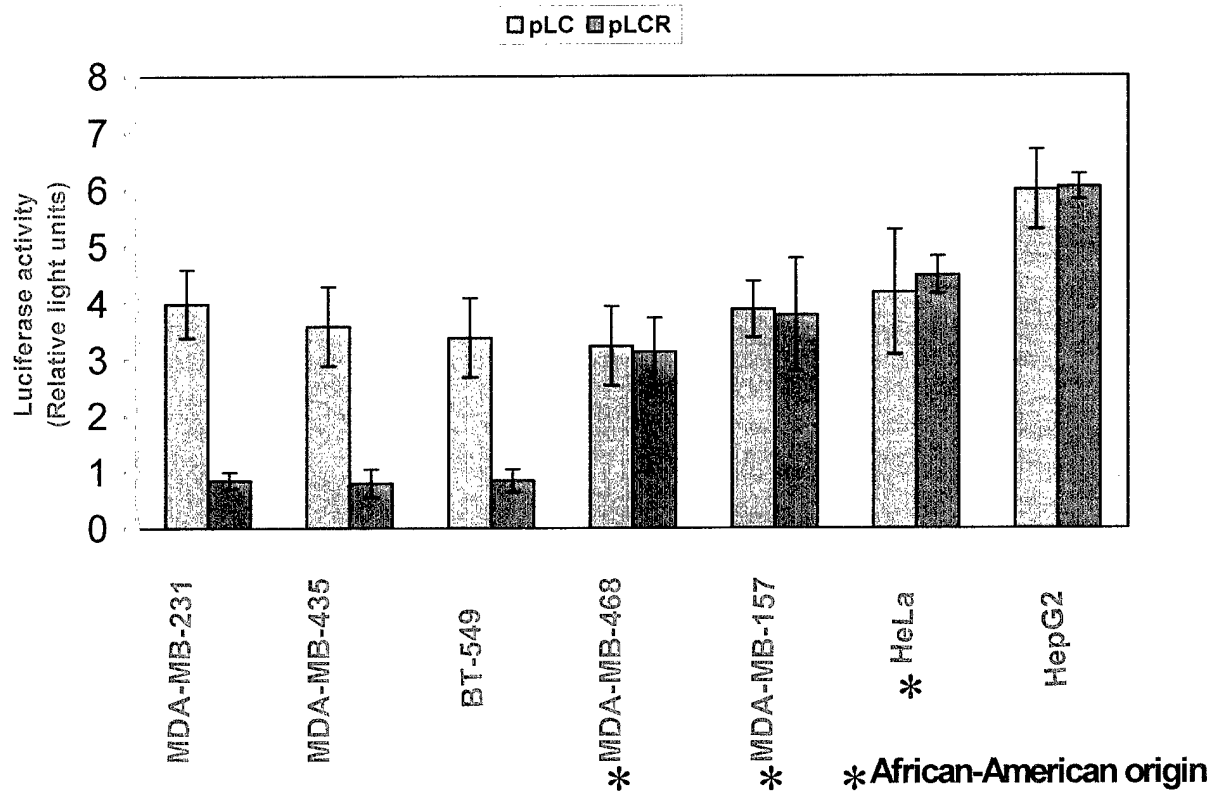


Fig. 6. Differential activities of the BRCA2 gene silencer in human breast cells and non-breast cells of Caucasian and African American origins. Reporter plasmid pLC contains SV40 promoter in front of the luciferase gene and pLCR contains the BRCA2 221 bp silencer in front of the promoter. Similar experiments were done to obtain similar data when BRCA2 promoter was used instead of the SV40 promoter (not shown).

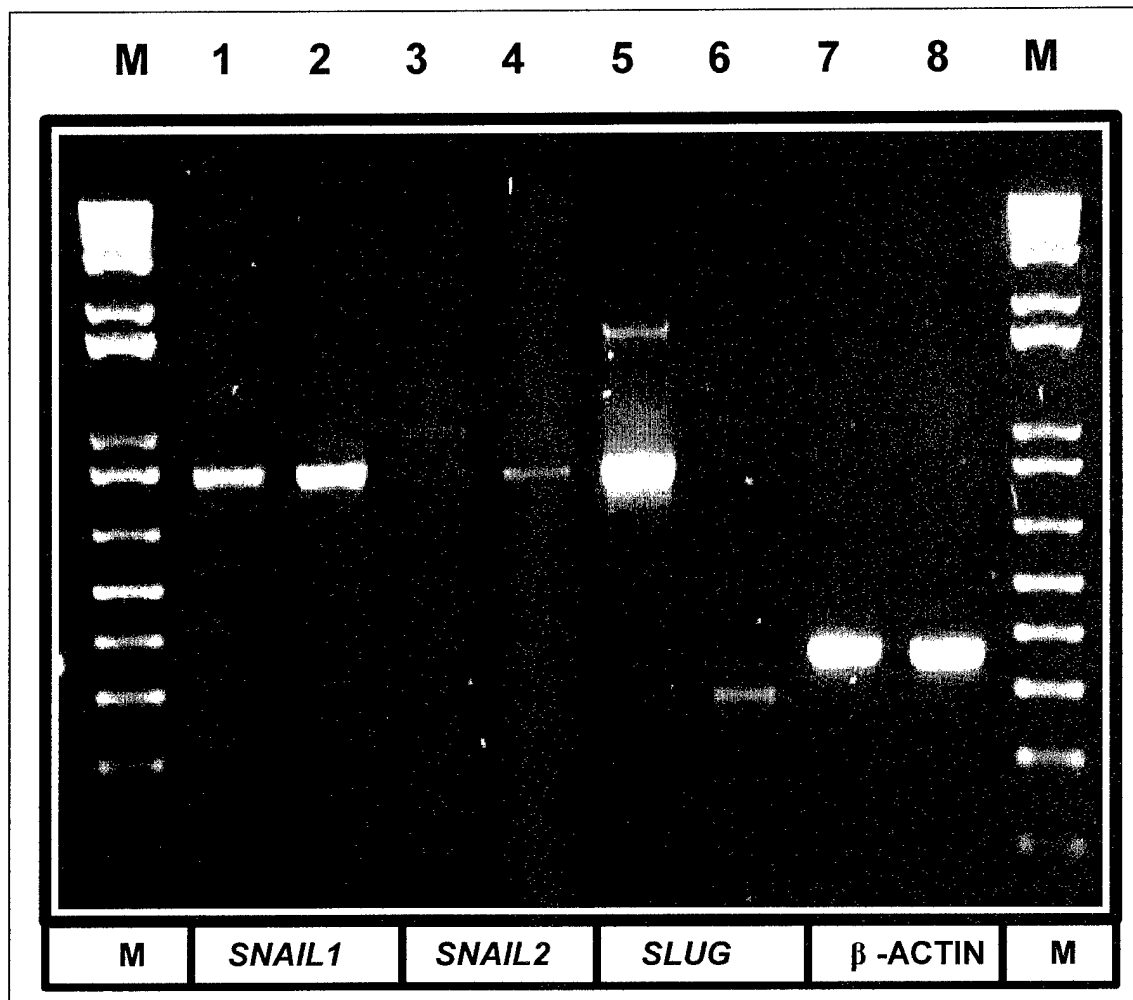


Fig. 7. RT-PCR analysis of the expression of SNAIL1, SNAIL2, SLUG and β -actin mRNAs in BT549 cells (lanes 1, 3, 5, 7) and MDA-MB-468 (lanes 2, 4, 6, and 8 cells). These results are confirmed by real-time RT-PCR experiments (not shown).

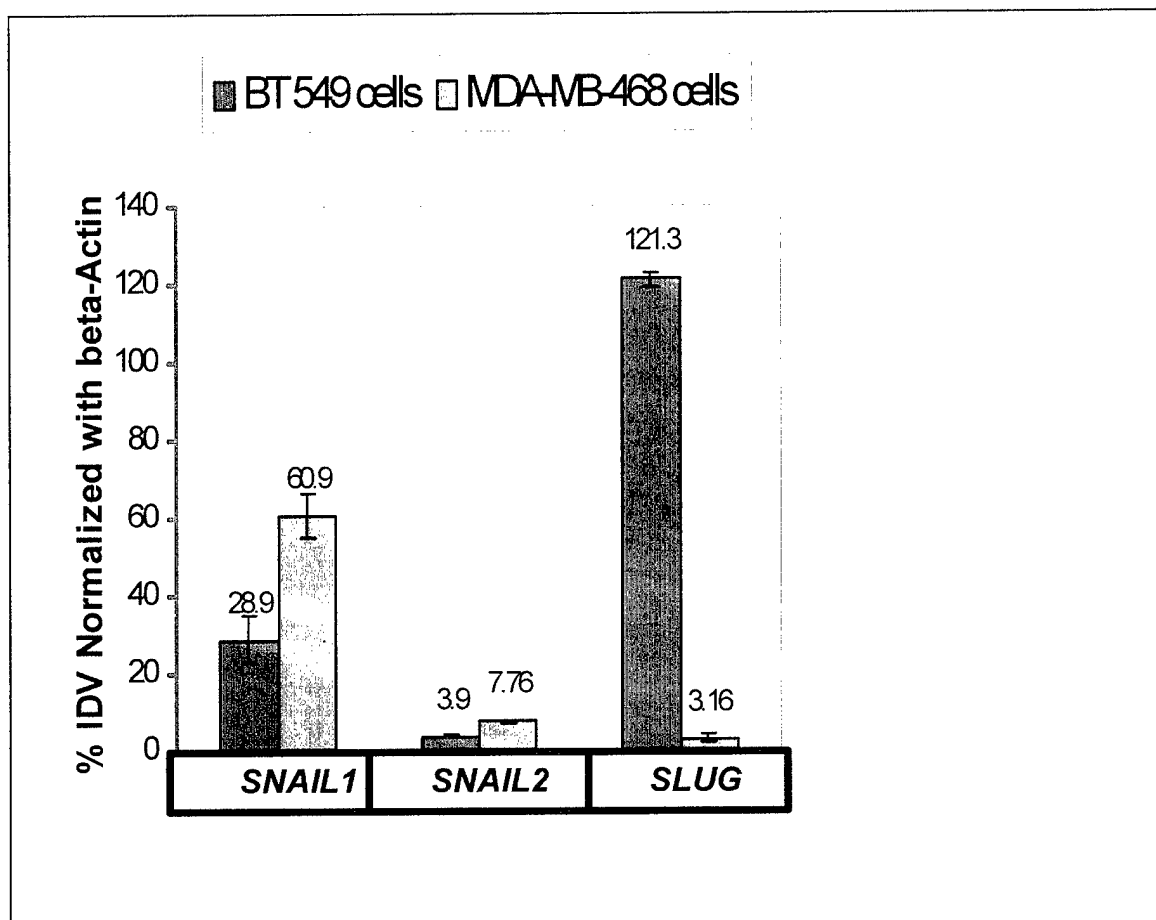


Fig. 8. Normalized data for RT-PCR analysis to evaluate the expression of SNAIL1, SNAIL2 and SLUG in BT549 and MDA-MB-468 cells.

Key Research Accomplishments

- A transcriptional silencer at the upstream [-701 to -921] of human BRCA2 gene was discovered. This 221 bp silencer sequence is composed of an E2-box in the middle of apparent binding sites for the Pol III transcriptional repressors ACR1 and YY1 and this cluster is flanked by two Alu-like sequences.

- This silencer is mainly active in the quiescent cells but not in the dividing breast cells.

- The E2-box and the flanking Alu-like sequences are essential for this silencer function.

- The ACR1/YY1 binding sites are needed for the de-silencing of this element in the dividing breast cells. The mechanisms of this activation and inactivation of this silencer in the quiescent and dividing cells, respectively, are presently unknown.

- Nuclear proteins from quiescent breast cell nuclear extract bind *in vitro* to the E2-box sequences.

- This silencer binds two proteins of 125 kDa and 29 kDa from quiescent cell nuclear extract.

- It binds a different protein of 19 kDa from dividing cell nuclear extract.

- Molecular sizes of these proteins suggest that they may belong to the E2-box binding zinc-finger transcriptional repressors of the *SNAIL* and *ZEB* family.

- The activity of the silencer in the African-American cells is much less than that in the Caucasian-American cells.

- The expression of the *SLUG* repressor is ~38 fold lower in the cells of African-American origin (MD-MB-468) in comparison to that from cells of Caucasian-American origin (BT-549).

Reportable Outcomes

Manuscripts in preparation

1. Tripathi, M.K. and Chaudhuri, G. (2004) Regulation of BRCA2 gene epigenetic silencer by *Snail* family of proteins. *J. Biol. Chem.* (In preparation).
2. Tripathi, M.K. and Chaudhuri, G. (2004) Differential activity of human BRCA2 gene silencer in breast cells of different ethnic origins (in preparation).
3. Tripathi, M.K. and Chaudhuri, G. (2004) Life without slug: Differential expression of cytokeratin genes in human breast cells that lack Slug gene expression (in preparation).
4. Khedkar, S. V., Tripathi, M.K. and Chaudhuri, G. (2004) Regulation of human BRCA2 gene expression by differential expression of hRFC1 gene (in preparation).

Poster presented

1. Misra, S., Tripathi, M.K. and Chaudhuri, G. (2002) Evaluation of the SLUG and the SNAIL gene expression in quiescent and dividing Human Mammary Epithelial Cells. Poster presented at Meharry Medical College/Vanderbilt Ingram Cancer Center (MMH/VICC) Alliance 2nd Annual Retreat, November 2nd 2002, Vanderbilt Ingram Cancer Center, Vanderbilt University, Nashville, TN- 37208, USA.

2. Tripathi, M.K. and Chaudhuri, G. (2002) H1 RNA Promoter-based expression of short hairpin RNAs (shRNAs) against BRCA2 transcripts in Human Mammary Epithelial Cells. Poster presented at Meharry Medical College/Vanderbilt Ingram Cancer Center (MMH/VICC) Alliance 2nd Annual Retreat, November 2nd 2002, Vanderbilt Ingram Cancer Center, Vanderbilt University, Nashville, TN- 37208, USA.
3. Daugherty, V.J., Tripathi, M.K. and Chaudhuri, G. (2003) DNA-Affinity purification of the nuclear proteins that bind to the transcriptional silencer of the human BRCA2 gene. Poster presented at 2003 AACR annual meeting (July 11-15) at Washington, D.C., USA.
4. Tripathi, M.K. and Chaudhuri, G. (2003) Regulation of human BRCA2 gene silencer by SLUG and SNAIL in quiescent cells. Poster accepted for presentation at 2003 AACR annual meeting (July 11-15) at Washington, D.C., USA.
5. Khedkar, S. V., Tripathi, M.K. and Chaudhuri, G. (2003) Effect of transient ablation of the folate receptor (RFC1) gene expression on the BRCA2 mRNA level in MCF7 cells. Poster presented at Meharry Medical College/Vanderbilt Ingram Cancer Center (MMH/VICC) Alliance 3rd Annual Retreat, Vanderbilt Ingram Cancer Center, Vanderbilt University, Nashville, TN- 37208, USA.
6. Farrow, Anitra L., Misra, Smita, Tripathi, Manish K. and Chaudhuri Gautam (2003) Bacterial Expression and Purification of the SLUG Protein from Human Breast Cells and Antisera Development. Poster presented at Meharry Medical College/Vanderbilt Ingram Cancer Center (MMH/VICC) Alliance 3rd Annual Retreat, Vanderbilt Ingram Cancer Center, Vanderbilt University, Nashville, TN- 37208, USA.
7. Khedkar, S. V., Tripathi, M.K. and Chaudhuri, G. (2004) Effect of siRNA-mediated knock down of reduced folate carrier protein on the expression of human BRCA2 gene. Poster accepted for presentation at 2004 AACR annual meeting at Orlando, FL, USA.

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Conclusions

In the United States, among all the women, breast carcinoma is the most frequently occurring cancer and the second leading cause of cancer death. Many environmental, reproductive, and genetic factors have been associated with an increased risk of breast cancer. The breast cancer susceptibility gene, BRCA2, is one of the pivotal genes for the regulated growth and proliferation of human breast and ovary cells. Among families of with the same BRCA2 mutations, there are differences in age specific penetrance, lifetime penetrance, proportions of breast cancer, and risk of other cancers. This variability suggests that there are

environmental and genetic factors interacting with the BRCA2 gene. Although Caucasian-American females have the highest incidence rates of breast carcinoma, African-Americans have the highest mortality rates. African-American females typically present with late-stage disease, the strongest prognostic factor for decreased survival. The focus of the proposed study is the human BRCA2 gene that is silenced in the resting breast cells but must be de-silenced and expressed in the dividing breast cells to obviate any DNA damage that occurs during cellular replication. Any malfunction of this de-silencing mechanism, for example by epigenetic means, thus, may lead to the onset of oncogenesis. The major goal of this study is to understand the underlying molecular mechanisms in the silencing of BRCA2 gene expression in quiescent breast cells and the inactivation of the silencing mechanism to express BRCA2 in the dividing cells, particularly in the context of ethnic source of the breast cells. Towards this goal, we have found a transcriptional silencer at the upstream [-701 to -921] of human BRCA2 gene. This 221 bp silencer sequence is composed of an E2-box in the middle of apparent binding sites for the Pol III transcriptional repressors ACR1 and YY1, and this cluster is flanked by two Alu-like sequences. This silencer is mainly active in the quiescent cells but not in the dividing breast cells. The E2-box and the flanking Alu-like sequences are essential for this silencer function. The ACR1/YY1 binding sites are needed for the de-silencing of this element in the dividing breast cells. The mechanisms of this activation and inactivation of this silencer in the quiescent and dividing cells, respectively, are presently unknown. Nuclear proteins from quiescent breast cell nuclear extract bind *in vitro* to the E2-box sequences. We purified two such groups of proteins from the nuclear extracts by DNA affinity chromatography. Molecular sizes of these proteins suggest that they may belong to the E2-box binding zinc-finger transcriptional repressors of the *Snail* and ZEB family. Our preliminary data show that the expression of the *Slug* repressor is 10-12 fold higher in the dividing cells (cancer or normal) of African-American origins in comparison to that from cells (cancer or normal) of Caucasian-American origins and the inactivation of the silencer in the African-American cells is much less than that in the Caucasian-American cells. Understanding the molecular mechanisms involved in the regulation of expression of BRCA2 may widen the horizon of the etiology of breast and ovarian cancer, particularly that of sporadic nature. The understanding of the regulation of BRCA2 gene expression with respect to the ethnic origin of the breast cells will also contribute to our understanding of ethnic disparity in breast cancer prognosis and disproportionate impact of this disease to African-American females.

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